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Integrated molecular systems for biosensors *

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Abstract

Redox enzymes have been assembled in a monolayer on a solid surface by a potential-assisted self-assembly method as well as a thiol–gold self-assembly method. These enzymes communicate electronically with the solid substrate through a molecular interface conducting polymer and a covalently bound mediator. An ordered antibody array has also been assembled on the solid surface by a combination of the Langmuir–Blodgett (LB) film method and the self-assembly method. An ordered monolayer of protein A is deposited on the solid surface by the LB method, which is followed by self assembly of antibody. Individual antigen molecules that are complexed with the antibody array have been quantitated selectively by atomic force microscopy (AFM). A TOL plasmid, encoding a xyl R binding protein for xyline and a firefly luciferase marker enzyme, has been implemented in a bacterial cell. The whole cell responds to environmentally hazardous substances such as xylene by emitting light.

Keywords: Biosensors; Integrated molecular systems

1. Introduction

Biosensing technology has made marked progress in the last decade, with great success in the implementation of biological selectivity in electronic and optoelectronic devices. Enzymes as well as antibodies have been the major proteins for molecular recognition in biosensing. However, several problems have remained unsolved. One of these is to fabricate an ordered protein molecular array on a solid surface. Secondly, several different functional proteins should be assembled on the solid surface while retaining their integrated network of individual molecular functionality in a similar manner to intercellular molecular networks. Thirdly, new technology for monitoring the physicochemical properties of individual molecules on the solid surface should be explored. These should be the targets of biosensor research and development in the coming decade.

Two different types of self-assembly processes are described here. One is the potential-assisted self assembly of redox enzymes on the surface of an electrode. The potential-assisted self assembly is followed by electrochemical deposition of a conducting polymer that works as a molecular interface to enable the redox enzyme to communicate electronically with the electrode. The other is a self assembly of mediator-modified redox enzymes on the surface of a porous gold electrode. In addition, the fabrication of antibody arrays on a solid surface by Langmuir–Blodgett (LB) film technology coupled with self-assembly technology will also be presented.

Most biosensing devices make use of protein molecules on solid surfaces and their sensing principles depend on the changes of the physicochemical properties after the corresponding molecular recognition. Because these physicochemical changes are derived from the averaging properties of the surfaces, such biosensing technologies have suffered from perturbations due to various artifacts. One of the ultimate goals of biosensing technology is to create a new technology to quantitate selectively individual molecules in a specific site. Atomic force microscopy (AFM) has been successfully applied to quantitate individual antigen molecules that are aligned on the ordered structure of the corresponding antibody protein array.

In the last part of this paper a novel application of a molecular network to a biosensing system will be described. The molecular network consists of a plasmid encoding a binding protein specific for environmentally hazardous compounds such as xylene and a marker

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enzyme, firefly luciferase. As the DNA encoding firefly luciferase is expressed by complexation of the binding protein with the environmentally hazardous compounds, the whole molecular network responds to environmentally hazardous compounds by emitting light. The molecular network has been implemented in a whole cell and fabricated in a biosensing device.

2. Potential-assisted self assembly and molecular interfacing of redox enzymes on the electrode surface

One of the key technologies required for fabricating biomolecular electronic devices concerns the molecular assembly of electronic proteins such as redox enzymes on a monolayer scale on the electrode surface. Furthermore, the molecularly assembled electronic proteins are required to communicate electronically with the electrode [1–4]. Individual protein molecules on the electrode surface should be electronically accessed through the electrode. To fulfil these requirements, two fabrication processes have been proposed by us. One is a potential-assisted self assembly of redox enzymes on the electrode surface, which is followed by electrochemical fabrication of a monolayer-scale conducting polymer on the electrode surface for molecular interfacing (Fig. 1). The other is self assembly of mediator-modified redox enzymes on a porous gold electrode surface through the thiol–gold interaction.

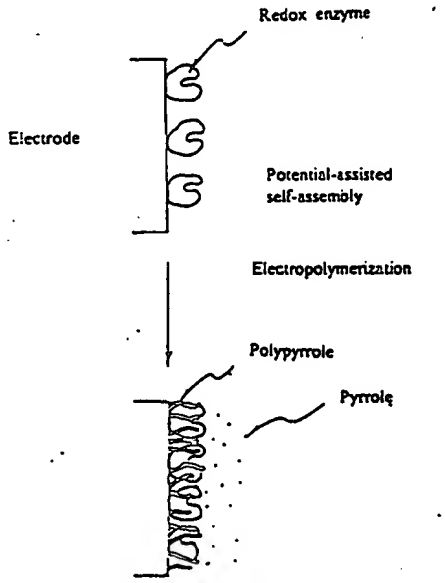

Fig. 1. Potential-assisted self assembly of redox enzyme on the electrode surface followed by electrochemical polymerization of pyrrole to fabricate the molecular interface between the enzyme molecule and the electrode.

The potential-assisted self assembly is carried out in an electrolytic cell equipped with a platinum or gold electrode (working electrode) on which a protein monomolecular layer is formed, a platinum counter electrode, and an Ag/AgCl reference electrode. The potential of the working electrode is precisely controlled by a potentiostat with reference to the Ag/AgCl electrode. A protein solution should be prepared taking the protein isoelectric point into account, because the protein becomes negatively charged in the pH range above its isoelectric point.

Fructose dehydrogenase (FDH) is a redox enzyme with pyrrollo-quinoline quinone (PQQ) as the prosthetic group. A monolayer of FDH was formed on the platinum or gold electrode surface by potential-assisted self assembly [5]. FDH was dissolved in pH 6.0 phosphate buffer to make its net charge negative. FDH molecules instantly adsorb on the electrode surface primarily due to electrostatic interaction. Under a controlled electrode potential, the FDH adsorption increased with time and reached a steady state. In the potential range 0 to +0.5 V, the adsorption rate of FDH increased sharply with electrode potential. FDH molecules may be self assembled on the electrode surface in such a manner that the negatively charged site of the FDH molecule faces the positively charged surface of the electrode. Enzyme assay clearly showed that electrode-bound FDH retained its enzyme activity without appreciable inactivation.

In the next step a molecular wire for the molecular interface was prepared for the electrode-bound FDH that was made by potential-assisted self assembly. Polypyrrole was used as the molecular wire of the molecular interface for the electrode-bound FDH and was synthesized by electrochemical oxidative polymerization of pyrrole.

Electronic communication between the electrode-bound FDH and an electrode has been confirmed by differential pulse voltammetry.

In addition to FDH, potential-assisted self assembly has been successfully applied to several redox enzymes including glucose oxidase and alcohol dehydrogenase. The self-assembled redox enzymes have also been molecularly interfaced with the electrode surface by a conducting polymer.

3. Self assembly of mediator-modified redox enzymes on a porous gold electrode surface

In contrast to the molecular wire of the molecular interface, electron mediators are covalently bound to a redox enzyme in such a manner that an electron tunnelling pathway is formed within the enzyme molecule. Therefore, enzyme-bound mediators work as a molecular interface between an enzyme and an elec-

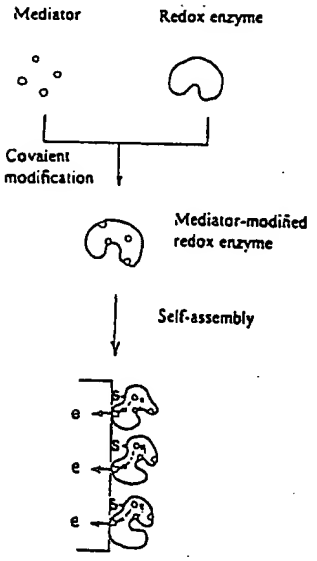

Fig. 2. Self assembly of mediator-modified enzyme on the porous gold-black electrode surface.

trode. Degani and Heller proposed the intramolecular electron pathway of ferrocene molecules covalently bound to glucose oxidase [6]. However, few fabrication methods have been developed to form a monolayer of mediator-modified enzymes on the electrode surface. We have succeeded in the development of a novel preparation of the electron-transfer system of a mediator-modified enzyme by self assembly in a porous gold-black electrode (Fig. 2).

Glucose oxidase (from *Asperigillus niger*) and ferrocene carboxyaldehyde were covalently conjugated by the Schiff base reaction, which was followed by $NaBH_4$ reduction. The conjugates were dialysed against phosphate buffer with three changes of buffer and assayed for their protein and ion contents. Porous gold-black was electrodeposited on a micro gold electrode by cathodic electrolysis with chloroaric acid and lead acetate. Aminoethane thiol was self assembled on a smooth gold-disk electrode (5 mm in diameter) and a gold-black electrode (100 μm in diameter). Ferrocene-modified glucose oxidase was covalently linked to either the modified plain gold or gold-black electrode by glutaraldehyde.

Note that the anodic peak current increases considerably with an increase in the molar ratio of ferrocene to glucose oxidase whilst the amount of enzyme self assembled on the electrode surface is fixed. This indicates that each modified ferrocene may contribute to electron transfer between the enzyme and the electrode in the case of the gold-black electrode, and the ferrocene-modified enzyme could form multi-electron transfer paths on the porous gold-black electrode.

The substrate concentration dependence of the response current of the gold-black electrode was compared with that of the gold-disk electrode. The ferrocene-modified glucose oxidase used in this measurement has 11 ferrocenes per glucose oxidase. The electrode potential was controlled at 0.4 V versus Ag/AgCl. The response current was recorded when the output reached a steady state. The response current was enhanced when ferrocene-modified glucose oxidase was self-assembled on a porous gold-black electrode.

The porous matrix of the gold-black electrode enabled the ferrocene-modified glucose oxidase to perform a smooth electron transfer by means of easy access between the self-assembled molecules and the electrode surface.

4. Self-assembled antibody protein array on protein A monolayer

Biosensors may be classified into two categories: biocatalytic biosensors and bioaffinity biosensors. Biocatalytic biosensors contain a biocatalyst such as an enzyme to recognize the analyte selectively. Bioaffinity biosensors, on the other hand, may involve antibody, binding protein or receptor protein, which form stable complexes with the corresponding ligand. An immunosensor in which antibody is used as the receptor may represent a bioaffinity biosensor.

Advanced biotechnology and monoclonal antibody production have provided strong support for bioaffinity biosensors, and various new principles of electrochemical and optical immunosensors have been proposed. Concentrated efforts have been sharply focused on the development of homogeneous immunosensors, which require no bound–free separation. Examples include an optical immunosensor based on surface plasmon resonance (SPR), an optical fibre immunosensor based on fluorescence determination using an evanescent wave and an optical fibre electrode immunosensor based on electrochemical luminescence determination. These immunosensors are characterized by a single step of determination and high selectivity as well as high sensitivity. The responses of these immunosensors, however, result from averaging the physicochemical properties of the antibody-bound solid surface. We have succeeded in fabricating an ordered array of antibody molecules on the solid surface and in quantitating individual antigen molecules that are complexed with the antibody array.

Protein A is a cell-wall protein of *Staphylococcus aureus* with a molecular weight of 42 000. Since protein A binds specifically to the Fc part of IgG from various animals, it has been widely used in immunoassay and affinity chromatography. We found that protein A could be spread over the water surface to form a monolayer

membrane by the LB method. On the basis of this finding, an antibody array on the solid surface can be obtained by the following two steps. The first step is fabrication of an ordered protein A array on the solid surface by the LB method. The second step is self assembly of antibody molecules on the protein A array by biospecific affinity between protein A and the Fc of IgG (Fig. 3).

A Fromhertz type of LB trough was used for fabrication of the protein A array on highly oriented pyrolytic graphite (HOPG) (15 mm×15 mm×2 mm). Protein A was dissolved in ultrapure water to make a 0.1×10^{-6} g ml^{-1} solution. With a micropipette, 0.2 ml of protein A solution was dropped on 150 cm^2 of the air/water interface of a compartment that contained ultrapure water as subphase. The protein A layer was compressed at a rate of 10 mm^2 s^{-1} with a barrier. Compression was stopped at a surface pressure of 11 mN m^{-1} and the monomolecular layer of protein A was transferred to an adjacent compartment containing 0.5% glutaraldehyde solution at a rate of 10 mm^2 s^{-1}. The protein A layer was incubated for 1 h to be crosslinked by glutaraldehyde, which was followed by transfer to a compartment containing ultrapure water for rinsing. The protein A molecular membrane was then transferred onto the surface of an HOPG plate by the horizontal method. The molecular imaging of the preparation was obtained by AFM in solution.

To prepare an antibody protein array, a monolayer of protein A, which was compressed at a surface pressure of 11 mN m^{-1}, was transferred to a compartment containing anti-ferritin antibody in 10 mM pH 7.0 phosphate buffer. The antibody molecules were self assembled onto the protein A layer. The protein A/antibody molecular membrane was transferred to a compartment containing ultrapure water for rinsing, and was then transferred onto the surface of an HOPG plate by the horizontal method. AFM measurements were made in a pH 7.0 10 mM phosphate buffer solution.

AFM imaging of the protein A array deposited on an HOPG plate showed an ordered alignment of protein molecules when the measurement was made in a pH 7.0 10 mM phosphate buffer at a controlled force of 4×10^{-11} N and a scanning rate of 0.6 Hz. However, an ordered structure was not observed unless the protein A molecules were not crosslinked by glutaraldehyde.

The antibody array that was self assembled on the protein A array was also visualized in molecular alignment by AFM. The antibody array was in contact with a pH 7.0 10 mM phosphate buffer. The AFM measurement was conducted at a controlled force of 1.8×10^{-11} N and a scanning rate of 0.5 Hz. The molecular size of the antibody was estimated as 7 nm in diameter.

The antibody array was soaked in different concentrations of ferritin solutions for 1 h, and was assayed for AFM imaging in solution. Ferritin molecules were recognized and fixed by the antibody array. The ferritin concentration was 10 ng ml^{-1}. Individual ferritin molecules on the antibody array were selectively quantitated by AFM imaging.

5. TOL-plasmid molecular network for bioluminescent sensing of environmental pollutants

To prevent environmental pollution, the spreading of pollutants, such as chemicals that are difficult to degrade, should be strictly kept as low as possible. Therefore, a rapid and sensitive detection method for environmental pollutants is required to prevent the pollutants from diffusing away to the environment. The recent development of molecular-based detection techniques has greatly increased the possibility of developing new materials in biosensing systems. The insertion of marker genes, such as the genes for enzymes and binding protein, allows various substances of biological impor-

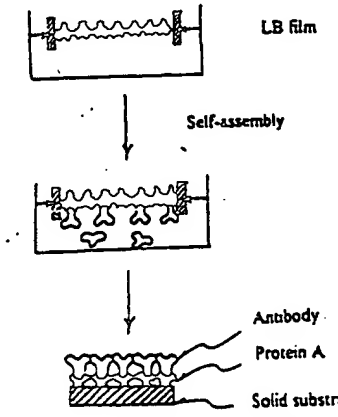

Fig. 3. Fabrication of self-assembled antibody protein array on a protein A monolayer formed by LB film technology.

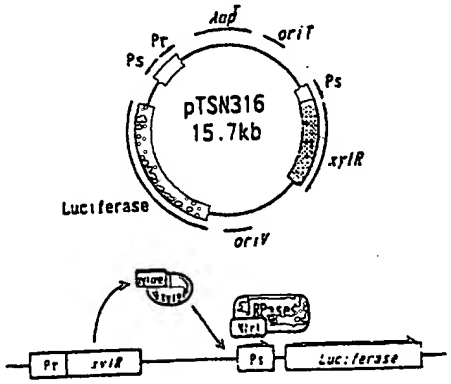

Fig. 4. Schematic illustration of a TOL-plasmid molecular network for bioluminescent sensing of environmental pollutants.

tance to be tracked. Although these fusion proteins have enormous implications for the study of protein engineering, the main impetus has been the potential technological benefits of genetically engineered microorganisms capable of demonstrating novel functions, such as the monitoring of industrial wastes in the environment. A variety of plasmids of the *Pseudomonas* species encode valuable enzymes for the degradation of a series of chemicals that are hard to assimilate, such as aromatic compounds and halogenated derivatives. Among them, the TOL plasmid is one of the interesting plasmids, as it carries a series of enzymes for the assimilation of benzene derivatives.

The introduction of luminescent enzymes enables specific microorganisms to detect industrial pollutants in situ, without extracting the marker enzymes. Bioluminescence-based techniques offer several advantages, such as the non-destructive detection of marked substances in sewage water and in soil samples. These techniques involve introduction of genes for the recognition of the marked substances and for luminescence generation, originally cloned from *Pseudomonas putida* and firefly, respectively. In the TOL plasmid, the product of xylR and xylS genes stimulates the transcription of the following genes by activating the promotor of the plasmid (Fig. 4).

Luminescent monitoring of environment pollutants by photon-generating microorganisms has been performed by a photon-counting device, since techniques for measuring photons have high sensitivity and provide a linear response over several orders of magnitude. In addition, a new sensing system for the protection of the environment has also been developed by immobilizing a luminescent microorganism of the surface on an optical fibre.

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